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Mediated by Cadherin T6

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13. ABSTRACT (Maximum 200 Words) This phase I project characterized a gene product initially referred to as "T6" that is upregulated when cultured prostate cancer cells were selected for an apoptosis-resistance phenotype. We have characterized this molecule and now call it protocadherin-PC. It is a very unusual species of protocadherin-family molecules in that it is localized cytoplasmically instead of membrane-bound (like other members of this family) and we have shown that it binds to the wnt-signaling transcription factor, β -catenin, activating transcription from β -catenin/TCF promoters. Thus, expression of protocadherin-PC in prostate cancer cells appears to activate the wnt signaling pathway in an analogous manner to which wnt signaling is activated during progression of colon cancer or melanoma. We have also found that this unique protocadherin is encoded on the human Y chromosome and we have shown that the Y-chromosomal form of protocadherin-PC is highly upregulated in actual specimens of hormone-resistant prostate cancers in conjunction with activation of the wnt-signaling pathway. Therefore, the results of this project suggest that activation of the wnt-signaling pathway by protocadherin-PC has an important role in the development of therapeutic resistance in prostate cancer patients.			
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INTRODUCTION

Advanced human prostate cancer rapidly develops resistance to hormones and other therapeutic agents used to treat it. In order to better understand the genetic changes that accompany the development of therapeutic resistance by prostate cancer cells, we have developed a unique set of human prostate cancer cell lines with a generic resistance to therapeutic agents and we have genetically compared these cell lines to the parental, therapeutic-sensitive cells, from which they were derived. These experiments enabled us to identify a partial cDNA sequence, originally referred to as T6, that is expressed in the therapeutically resistant cell lines but not in the parental prostate cancer cells. Preliminary characterization of this gene product showed that it was a new member of the cadherin gene family, a family of gene products that are thought to be important for intracellular adhesion as well as involved in cellular signaling processes. Our application proposed the full characterization of the T6 gene product by complete sequencing of the cDNA, identification of T6 gene structure and the development of antibodies against the protein encoded by the T6 cDNA. These goals have been totally accomplished during the project period and this work has revealed the unique and unusual nature of this apoptosis-regulating gene product that has now been renamed "Protocadherin-PC". In addition we have been able to identify a putative mechanism through which the protocadherin-PC molecule might alter prostate cancer cell sensitivity to therapeutic agents ; apparently this molecule promotes cellular signaling through the wnt signaling pathway (mediated by the transcription factor, β -catenin) and our findings indicate that prostate cancer cells can progress to hormone resistance through a mechanism that is similar to that involved in the progression of human colon cancers and melanomas. We have also developed the means to survey human prostate cancer specimens for expression of this gene product and our preliminary survey indicates that protocadherin-PC is highly expression in actual hormone-resistant human prostate tumors and that the expression of protocadherin-PC in these tumors correlates with abnormalities in β -catenin localization. Finally, we have identified the chromosomal location of the gene encoding protocadherin-PC and, interestingly, it is on the Y-chromosome, a chromosome that frequently demonstrates anomalies in prostate cancer cells.

Body

Task 1. To fully characterize the human gene encoding T6 as well as its mRNA and protein products.

This project originated from studies in which we derived apoptosis resistant variants of the LNCaP cell line by sequential repeated exposure of parental LNCaP cells to one of two different agents that induce apoptosis of these cells (phorbol esters or serum starvation) followed by expansion of the surviving cell populations.

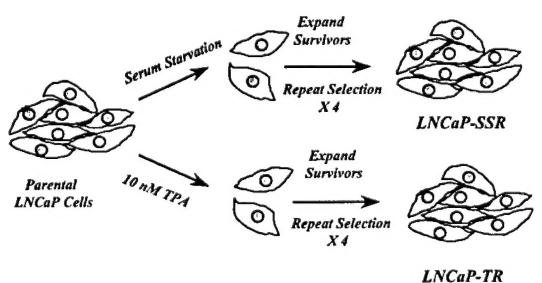


Figure 1. Scheme for derivation of apoptosis-resistant LNCaP derivatives

The two resistant cell lines, referred to as LNCaP-TR (for TPA resistant) and LNCaP-SSR (for serum starvation resistant) were shown to be resistant to the alternate agent that was not used in their selection (i.e. LNCaP-TR cells were found to be resistant to serum starvation and LNCaP-SSR cells were resistant to phorbol ester) (Figure 2) and they were also shown to be hormone resistant by testing for their ability to form tumors in castrated male immunodeficient mice following xenografting (LNCaP-TR cells form tumors in 9/10 xenografted castrated male nude mice whereas parental LNCaP cells do not form tumors in such mice). Thus they were found to be generically “apoptosis-resistant” when compared to the parental LNCaP cells from which they were derived.

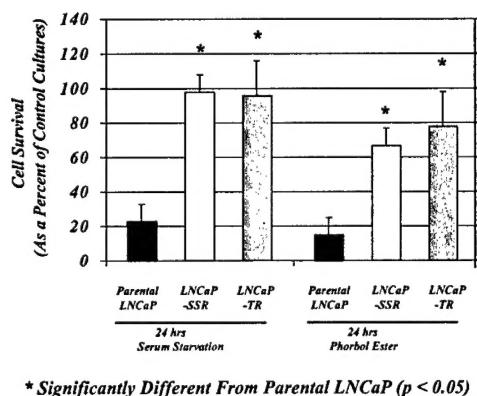


Figure 2. Survival of LNCaP Variants Following 24 hr exposure to apoptotic agents.

A subtractive hybridization-PCR technique was then used to comparatively screen for genetic differences between LNCaP-TR and parental LNCaP cells. This screening technique enabled us to identify a 249 bp fragment of cDNA, that we initially referred to as “T6”. Hybridization of this cDNA fragment to a Northern blot demonstrated that an approximate 4.6 kb mRNA homologous to the probe is highly expressed in the LNCaP-TR as well as the -SSR cells and also demonstrated that this transcript is highly upregulated in the parental LNCaP cells following exposure to a testosterone-deficient medium.

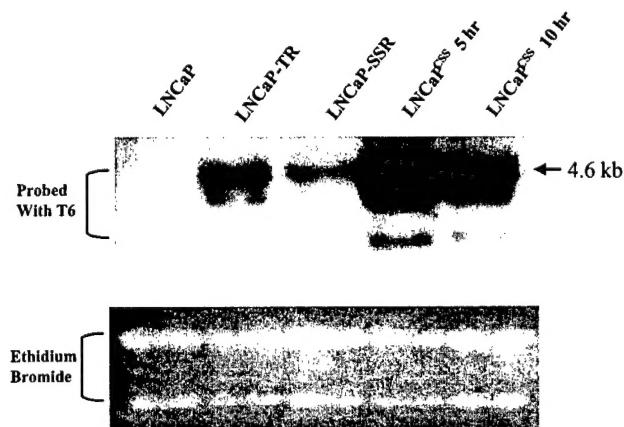


Figure 3. (Upper Panel) Hybridization of the 249 bp T6 cDNA probe to a Northern blot containing 25 ug RNA extracted from LNCaP parental cells, LNCaP-TR, LNCaP-SSR or parental LNCaP cells grown in

charcoal-stripped serum-containing medium (LNCaP^{css}) for 5 or 10 hrs. The probe recognizes a predominant 4.6 kb mRNA (in addition to some minor species) that is highly expressed in -TR and -SSR cells and is induced in parental LNCaP in response to testosterone-free medium. (Lower Panel) Ethidium-bromide staining of Northern blot demonstrates similar loading of rRNAs.

An RNase protection assay (Figure 4) also showed that this message was also highly induced in LNCaP tumor xenografts when they are selected for their ability to grow in a testosterone-deficient environment following castration of the host mouse. This demonstrates that the gene product corresponding to the T6 probe is naturally upregulated as prostate cancer cells (LNCaP) are selected for the development of hormonal resistance in this *in vivo* animal model.

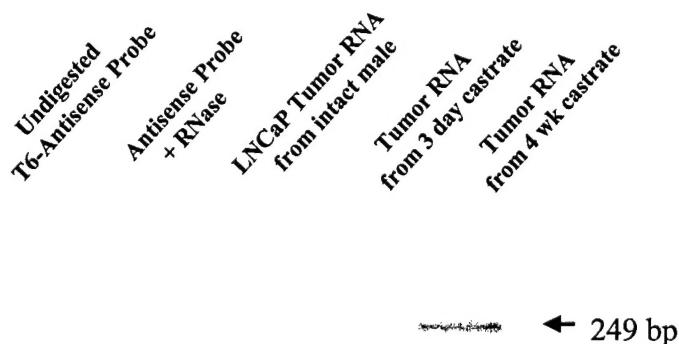


Figure 4. RNase Protection Assay of RNAs extracted from LNCaP tumors formed in immunodeficient-male mice. Results show that protection of the 249 bp T6 cDNA is elevated 25-fold or greater following 4 weeks of castration when the tumor has acquired hormone-insensitive characteristics.

In this first year of the project, sequence information from the T6 tag fragment was used to generate PCR primer sets for 5' and 3' RACE procedures to obtain the complete sequence of the homologous cDNA. These procedures resulted in the cloning of complete 5' and 3' cDNA fragments, each of which were sequenced by standard dideoxynucleotide techniques. Analysis of the complete sequence for the 4.8 kb cDNA (Figure 5) showed the presence of one long open reading frame of 2,868 bases that would translate to a polypeptide with 956 amino acids with a predicted molecular weight of approximately 105.9 kd. The predicted translation product would have 7 sequential regions that are characteristic of cadherin box domain sequences near the N-terminus (underlined in Figure 5). This cDNA thus identifies a new member of the cadherin superfamily of gene products and we have named it protocadherin-PC based upon the multiple nature of the cadherin box domains (more than 5) which is a proposed characteristic of the cadherin subfamily referred to as protocadherins (1) and because computerized (Fasta) searches of Genpept hit several protocadherins with E less than 0.001, but not classical cadherins with that low an E value. Protocadherin-PC also contains a serine-rich region near the C-terminal with partial homology to the β -catenin-binding site found on several classical cadherins (2). Another unusual aspect of the translation product of T6 is its lack of a signal sequence. This deficiency

makes it unlikely that the protocadherin-PC polypeptide would get through the endoplasmic reticulum and into the cell membrane, like most other members of the cadherin gene family.

A 15 amino acid peptide corresponding to residues 829-843 of the predicted protocadherin-PC polypeptide was synthesized and used to repeatedly immunize rabbits. Serum from immunized rabbits was used to probe a Western blot that contained proteins extracted from parental LNCaP cells and from the -TR and -SR variants. This serum specifically recognized a protein of approximately 110 kd on Western blots that was present in abundance in the apoptosis-resistant LNCaP variants but was only present in trace amounts in the parental LNCaP cells (Figure 6). A similar sized protein recognized by the antiserum was found to be abundantly present in extracts made from LNCaP tumor xenografts obtained after castration of the host mouse, but not before (Figure 6).

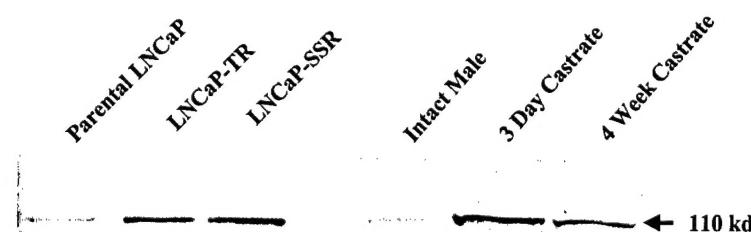


Figure 6. Western blot analysis of proteins extracted from parental LNCaP, LNCaP-TR or LNCaP-SR cells or from LNCaP Tumor xenografts removed from an intact male mouse or at 3 days or 4 weeks after castration demonstrates presence of 110 kb protein reactive with antibody made against predicted protocadherin-PC peptide.

Cellular fractionation showed that this 110 kd antigen was cytoplasmically localized (Figure 7) and together, these observations support the likely identify of the 110 kd band on Western blots as the protein product encoded by the protocadherin-PC transcript.

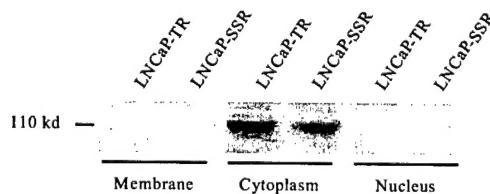


Figure 7: Western blot analysis for cellular localization of protocadherin-PC protein in fractionated extracts from LNCaP-TR or LNCaP-SSR cells. Results show the presence of the 110 kd reactive protein in the cytoplasmic extract of these cells, as would be predicted from its sequence.

In order to confirm the potential for β -catenin binding by the protocadherin PC protein, we directly immunoprecipitated protocadherin PC from cytosolic extracts of the LNCaP cell variants and then tested these immunoprecipitates by means of Western blotting to determine whether β -catenin protein was co-precipitated. The results of the Western blot (Figure 8) showed that the 94 kd β -catenin protein was present in the immunoprecipitates of the -TR and -SSR cells but not from the parental LNCaP cell that express low levels of protocadherin PC mRNA.

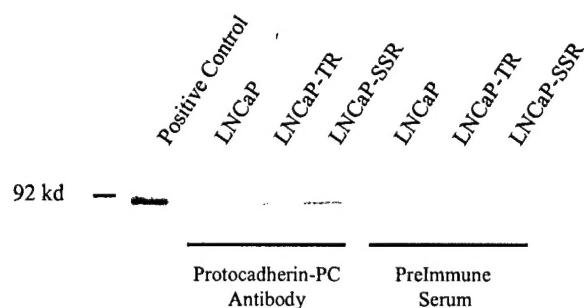


Figure 8. Co-Immunoprecipitation of β -catenin and protocadherin-PC. Antibody against protocadherin-PC (or Preimmune serum) was used to immunoprecipitate protocadherin-PC from LNCaP Cell Derivative Extracts. Proteins in the immunoprecipitates were electrophoresed and transferred to a Western blot. The Western blot was probed with anti- β -catenin antibody (94 kd).

Cadherin-family proteins are usually found in the outer membrane where they function to mediate intracellular adhesion between epithelial cells (3). The cytoplasmic domains of the classical cadherins are also known to directly or indirectly bind to several different proteins referred to as catenins. These proteins can influence the structural integrity of a cell (α -catenin anchors actin filaments to the cell membrane) or participate in the regulation of gene expression through the Wnt/Wingless signaling pathway (β -catenin is a transcriptional regulatory protein that complexes with the Lef-1/Tcf family of transcription factor proteins) (3). In normal epithelium, β -catenin is tightly retained at its cadherin binding site on the membrane. Any free cytoplasmic β -catenin is rapidly degraded by a complex reaction controlled by the Adenomatous Polyposis Coli (APC) protein (4). In colon and some other cancers, mutations in the APC protein or in β -catenin can stabilize β -catenin so that it accumulates in the cytoplasm and nucleus where it stimulates the expression of growth and survival proteins of these cells (5). Given this complex relationship and the potential for disruption of such a relationship by a new cytoplasmically-localized cadherin-related protein with β -catenin binding capacity, we surveyed our cell lines for the expression and compartmentalization of the cadherin-binding proteins, α - and β -catenin.

Parental LNCaP cells and the -TR and -SSR derivatives were fractionated into membrane, cytoplasmic and nuclear fractions and the proteins in these fractions were electrophoresed on an SDS-polyacrylamide gel and then transferred to a nitrocellulose filter. The Western blots were probed with antibody against E-cadherin, or against α - or β -catenin. As shown in Figure 9, E-cadherin in each of the cell derivatives was totally restricted to the membrane fractions, as should be expected. Comparison of

E-cadherin levels between the various lines did appear to show a reduction in this cellular adhesion and metastasis-suppressing protein in the apoptosis-resistant variants, -SSR and -TR.

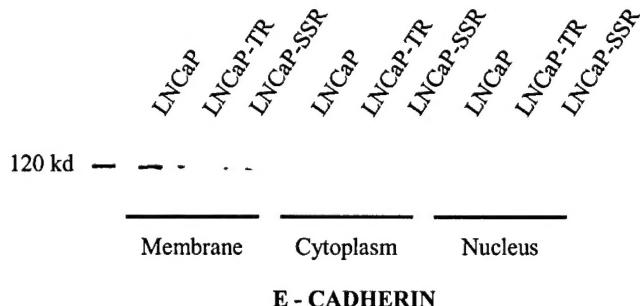


Figure 9. Confirmation of the membrane-specific localization of E-cadherin in all LNCaP cell derivatives. Cells were separated into membrane, cytoplasmic or nuclear fraction. Proteins were electrophoresed and transferred to a Western blot. The blot was probed with anti-E-cadherin

α -catenin, a structural protein, appeared to be equally expressed amongst the different cell lines and equally distributed between the membrane and the cytosolic fraction of each cell line (Figure 10).

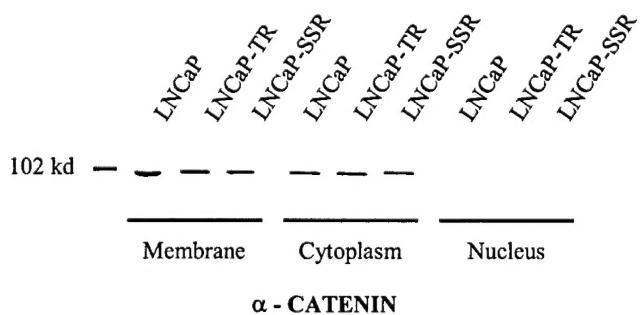


Figure 10. Localization of α -catenin expression in LNCaP cell derivatives. Extracts of membrane, cytoplasmic or nuclear fractions of cells were electrophoresed and a Western blot was probed for α -catenin using a specific antibody.

In contrast, although overall β -catenin levels were found to be similar in the 3 variant cell lines, the distribution of β -catenin protein was found to be remarkably different amongst the lines. In the parental LNCaP cells, β -catenin was exclusively retained in the cell membrane fraction, whereas in the -SSR line, most of the β -catenin is associated with the cytoplasmic fraction and in -TR cells the nuclear

fraction (Figure 11). This finding demonstrates that enhanced expression of the protocadherin-PC in the LNCaP-TR and -SSR cell lines is also associated with a remarkable change in the intracellular distribution of β -catenin, a protein which has the distinct potential of enhancing cellular survival when its nuclear transcriptional activity is facilitated.

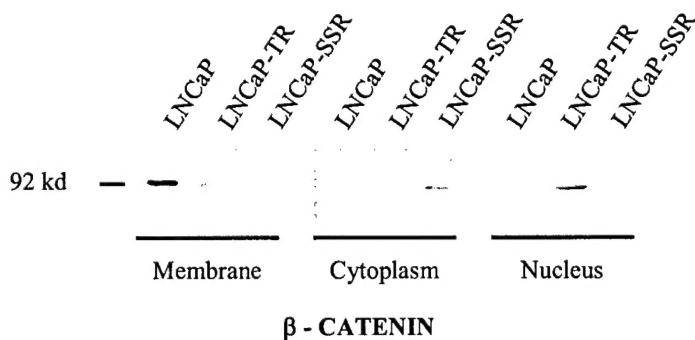


Figure 11. Abnormal localization of β -catenin in cytoplasm and nuclear fractions of LNCaP-TR and -SSR cells. The 94 kd protein is detected in nuclear and cytoplasmic fractions of extracts of the apoptosis-resistant cells but not the parental cells

The altered intracellular distribution of β -catenin that we detected in the apoptosis-resistant cell lines was also found to be associated with a coordinate increase in endogenous transcriptional activity from the Lef-1/Tcf transcriptional response element in these cells. When the LNCaP cell variants were individually co-transfected with a luciferase reporter plasmid containing Lef-1/Tcf response elements (pTOP-Flash) (6, 7) and a β -galactosidase reporter plasmid promoted by a simple CMV promotor element, the ratio of luciferase/ β -galactase activity was found to be over 2-fold greater than parental LNCaP in the -SSR line and over 4-fold greater than LNCaP in -TR line (Figure 12). Likewise, when we compared expression of luciferase reporter in these lines after transfection with pTOP-Flash (promoted by the optimal Lef-1/Tcf response element) or pFOP-Flash (luciferase reporter promoted by a mutated, inactive Lef-1/Tcf response element) (6, 7) we found similar results; i.e. a greater than 2-fold increased ratio of pTOP/pFOP luciferase activity in the -SSR cells and a greater than 2.5-fold increased ratio of pTOP/pFOP luciferase activity in the -TR cells (Figure 13). These results demonstrate that endogenous transcriptional activity promoted by the Lef-1/Tcf response element is significantly greater in the protocadherin-PC expressing LNCaP lines, -TR and -SSR than in the parental, apoptosis-sensitive LNCaP line and support the concept that the β -catenin-regulated transcriptional pathway is more active in the -SSR and -TR lines that express protocadherin-PC.

Figure 12 (Below) Standardized (to beta-galactosidase) expression of luciferase following transfection of LNCaP variants with p-TOP reporter plasmid (contains promotor elements for β -catenin/TCF transcription factor. Expression is significantly higher in -SSR and -TR cells than in parental LNCaP.

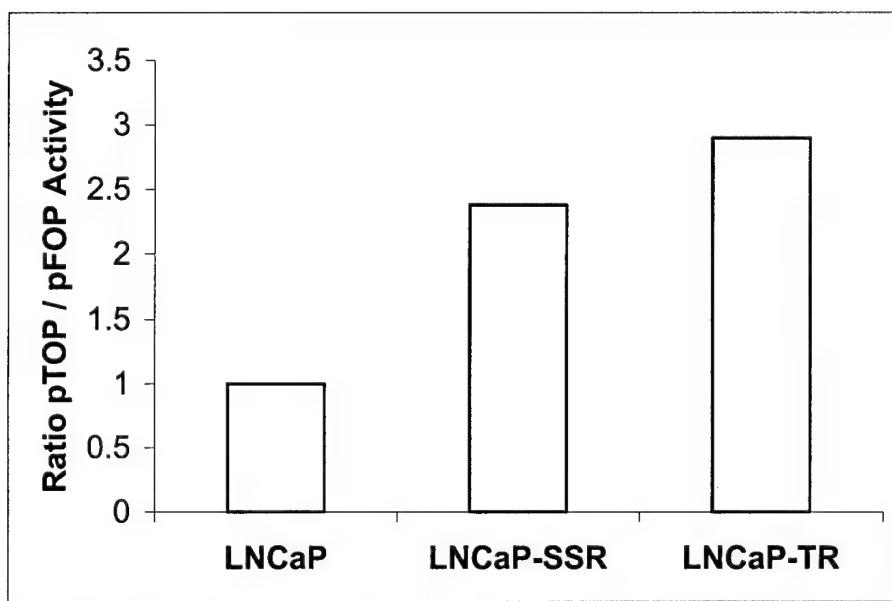
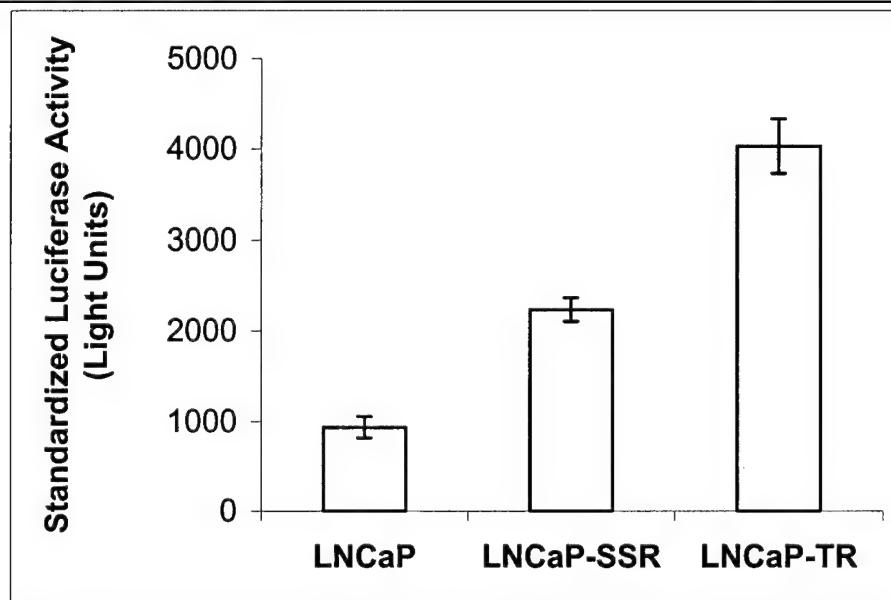


Figure 13 (Above) Relative expression of luciferase reporter when LNCaP-cell variants were transfected with TCF-sensitive pTOP or TCF-insensitive pFOP reporter plasmids. Data demonstrates that expression from the pTOP plasmid is significantly elevated in the apoptosis-resistant -SSR and -TR variants when compared to parental LNCaP and indicates increased activity of the wnt signaling pathway in these cells.

In a search of genbank for homologous sequences to protocadherin-PC cDNA, we identified a human genomic entry that had sequences with virtual complete homology to protocadherin-PC. These sequences were present in a contiguous DNA fragment isolated from the human X-chromosome (Accession # ac004388). Comparison of the genomic sequence to the cDNA sequence of protocadherin-PC allowed us to map the putative intron/exon structure of the protocadherin-PC-like cDNA present in the X chromosomal fragment. This comparison defined a potential protocadherin-PC-like gene product with 7 distinct exons that differed from protocadherin-PC cDNA only within the fifth exon, wherein an extra contiguous 13 bp sequence is present that is not found in the sequence of the protocadherin-PC cDNA isolated from the LNCaP cell derivatives. Computer analysis of the potential open reading frames encoded by the protocadherin-PC-like gene structure present on the X-chromosomal fragment (with the 13 bp addition) showed that the putative transcript from this gene contained one long open reading frame that would also translate into a cadherin-related protein. However, although this polypeptide retains complete homology to the amino acid structure of the prostate cancer protocadherin-PC throughout most of its carboxy terminal domain, it differs significantly from the polypeptide encoded by the protocadherin-PC transcript cloned from prostate cancer cells at its N-terminal domain, having a signal sequence that would enable it to be inserted into membranes. This is caused by a frame shift introduced by the extra 13 bp that affects the amino acid coding potential of the 5' region of the transcript and enables the use of an alternate methionine start further upstream of the first potential methionine encoded by the protocadherin-PC transcript that lacks the 13 bp sequence.

A more recent resurvey of genbank revealed a new entry (human genomic DNA) that contains the complete truncated (cytoplasmic) form of protocadherin-PC on a contiguous human DNA fragment derived from the Y-chromosome (accession #ac010722). Thus the 2 different forms of protocadherin-PC appear to be near-duplicate versions of the same gene, located on different human sex chromosomes. The truncated (cytoplasmic) form, encoded on the human Y chromosome, is the form that is expressed in abundance in the apoptosis-resistant prostate cancer cell derivatives and in hormone-resistant human prostate tumors. As well, this is likely to be the form of protocadherin-PC that has the capability of activating the β -catenin signaling pathway.

This can also be seen from the following experiment, in which we designed PCR primers to amplify the specific cDNA region containing the 13 bp discrepancy between the X- and Y-encoded protocadherin-PC like genes. When we used this primer set to amplify cDNA from the various LNCaP cell derivatives, (Figure 14), we found that the smaller cDNA product (specific to the Y-chromosomal gene lacking the 13 bp) was highly upregulated in the apoptosis-resistant -TR and -SSR cells compared to parental LNCaP

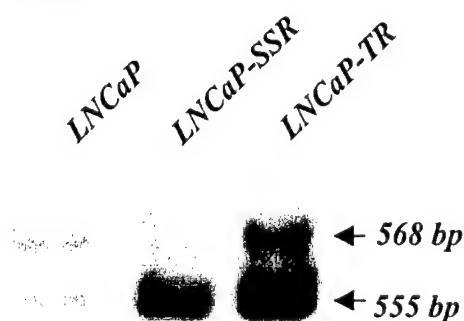


Figure 14. RT-PCR amplification of RNA extracted from parental LNCaP, LNCaP-SSR or LNCaP-TR cells using a primer set that spans the 13 bp discordant region between the proto-cadherin-PC like genes encoded on the X (568 bp) or Y (555 bp) chromosome. Results show that it is the Y-chromosome encoded version of the gene that is upregulated in apoptosis-resistant cells.

As well, we have now performed a preliminary survey of actual human prostate cancer tissues obtained from patients using a similar set of RT-PCR primers that distinguish the X- or Y-encoded gene. Our results, shown in Figure 15 below, confirm the idea that the Y-encoded version of protocadherin-PC becomes highly upregulated in the natural progression of prostate cancer to the hormone-insensitive state.

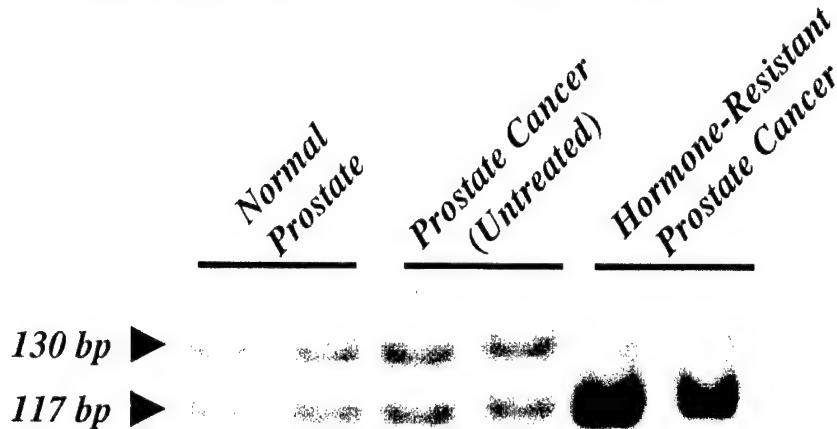


Figure 15. RT-PCR amplification of RNAs extracted from individual specimens (microdissected) of normal human prostate, untreated prostate cancer or hormone-resistant prostate cancer using another set of RT-PCR primers designed to amplify the discordant region between the proto-cadherin-PC like genes encoded on the X (130 bp) or Y (117 bp) chromosome. Results show large upregulation of Y-encoded gene product in hormone-resistant prostate cancers.

Task 2. *To determine whether genetic manipulation of cultured prostate cancer cells so that they overexpress T6 (Now Protocadherin-PC) cDNA diminishes their responsiveness to apoptotic agents in vitro and in vivo.*

We are still in the process of completing this task. Although we were able to derive the complete sequence of the Protocadherin-PC transcript based on 5' and 3' RACE cloning techniques, it proved more difficult to PCR the complete 4.8 kb cDNA, however this has now been accomplished. We have cloned the complete Protocadherin-PC cDNA fragment into several different expression vectors and expect to complete this task during the second Phase of the project.

Task 3. *To determine whether T6 (now Protocadherin-PC) expression influences the metastatic phenotype of prostate cancer cells.*

Completion of this task requires protocadherin-PC-transformed LNCaP cells which is in process of being completed at this time.

Task 4. To evaluate clinical specimens of human prostate cancer to determine whether T6 (Now Protocadherin-PC) expression correlates with therapeutic resistance of clinically aggressive prostate cancer.

In order to evaluate whether protocadherin-PC might be involved in the natural progression of human prostate cancers, we utilized a semi-quantitative RT-PCR assay (8) to evaluate expression levels of protocadherin-PC mRNA in RNAs extracted from 64 different human specimens consisting of normal or diseased (benign and malignant) human prostates. The assay compares the relative amplification efficiency of a small internal fragment of protocadherin-PC (260 bp) cDNA to the amplification of a fragment of a constitutive transcription factor cDNA (TBP). This assay, when applied to cDNAs from the LNCaP cell variants, confirmed the significant upregulation of protocadherin-PC mRNA expression in the -TR and -SSR variants and showed that cultured normal human prostate cells (epithelium or stroma) lacked protocadherin-PC expression (Not shown here). When the assay was applied to RNAs extracted from human prostate tissues, the results showed that there was low-level expression of protocadherin-PC-related mRNA in all normal prostate tissues, regardless of the anatomical zone of the prostate from which they were derived (peripheral, central or transitional zone), measured as a mean relative expression of 0.302 ± 0.169 ; 0.411 ± 0.119 ; or 0.231 ± 0.134 , respectively. A similar low level expression was maintained in specimens of diseased prostate tissues consisting of benign prostatic hyperplasia (BPH) or untreated (localized) prostate cancers (mean relative expression 0.287 ± 0.131 in BPH and 0.196 ± 0.204 in cancer). However, in contrast, tumors obtained from prostate cancer patients that were experiencing disease progression in spite of hormonal therapy (hormonal failure) were found to have a mean expression of protocadherin-PC mRNA that was significantly greater than any of the other types of tissues or tumors (mean relative expression = 1.031 ± 0.896 ; $p < 0.05$). This difference in protocadherin-PC mRNA expression was also found when tissue sections containing untreated prostate cancer or hormone resistant prostate cancer were compared following *in situ* hybridization for protocadherin-PC (not shown here). Significantly more intense hybridization was observed in the cancerous cells of all tumors obtained from patients with 6 months or more of hormonal therapy than was found in cancer cells in tumors from untreated patients. This data supports the results of our semi-quantitative RT-PCR assay and suggests that prostate cancer cells that survive after prolonged hormonal deprivation therapy of prostate cancer patients express much higher levels of protocadherin-PC-related mRNA when compared to prostate cancer cells that are therapeutically-naïve. Since the normal prostate tissues were found to express a low level of protocadherin-PC-related transcript, we also surveyed several other normal human tissues (brain, heart, liver, muscle, kidney, duodenum, placenta) using the same semi-quantitative RT-PCR assay. This survey showed that protocadherin-PC-related transcripts could also be detected as well in RNAs extracted from normal human brain (protocadherin-PC/TBP = 3.76) and placenta (protocadherin-PC/TBP = 2.87).

Using a primer set that would specifically amplify a small region within the putative fifth exon of protocadherin-PC (spanning the site containing the 13 bp cassette), we performed RT-PCR on cDNA of normal human brain or human placenta. The primers amplified only a single band of 130 bp from each of these tissues, that, when sequenced, proved to be the cDNA form of protocadherin-PC containing the 13 bp insertion that confers a membrane-binding potential on the translated protein. In contrast, when these primers were utilized to amplify cDNA extracted from the LNCaP cell variants or from normal human prostate or human prostate tumors, there were typically two amplification products, one at 130 bp and another at 117 bp (Figure 15, above). When the different sized amplification products were sequenced

they proved to be the two different forms of protocadherin-PC, with or without the 13 bp insertion. Moreover, it was clear from the abundance of the different forms of the RT-PCR products amplified from the prostate cancer cells and human prostate tissues/tumors that it is the expression of the truncated (cytoplasmic) form of protocadherin-PC mRNA that is increased in abundance when the LNCaP cells were selected for apoptosis-resistance or when primary human prostate cancers acquire hormone resistance (Figures 14, 15).

Key Research Accomplishments

- The complete cDNA sequence of Protocadherin-PC (Formerly T6) has been obtained
- The translation product of Protocadherin-PC has been described
- A polyclonal antibody was made that recognizes the Protocadherin-PC protein on Western blots
- The Protocadherin-PC protein was found to be localized within the prostate cancer cell cytoplasm
- The Protocadherin-PC protein was shown to functionally bind to β -catenin protein
- Apoptosis-resistant LNCaP cell variants that express Protocadherin-PC were found to have abnormal β -catenin localization (cytoplasmic and nuclear instead of membrane-bound)
- Apoptosis-resistant LNCaP cell variants that express Protocadherin-PC were found to have enhanced signaling through the β -catenin pathway
- The Protocadherin-PC gene was mapped to the human Y-chromosome (Yq11)
- A variant gene highly related to Protocadherin-PC was mapped to the X-chromosome (near Xq24)
- The X-chromosomal Protocadherin-PC-like gene was found to be membrane bound (in contrast to Protocadherin-PC which is cytoplasmically localized) and is expressed normally in human brain and placenta
- A survey of actual human prostate cancer specimens demonstrates that Protocadherin-PC expression is low in normal prostate, benign prostate hyperplasia and untreated prostate cancer but is significantly higher in hormone-resistant prostate tumors
- Coordinate analysis for expression of Protocadherin-PC and its homologue on the X-chromosome in prostate cancer specimens demonstrate that it is the truncated, cytoplasmic form of Protocadherin-PC (from the Y-chromosome) that is upregulated in hormone-resistant prostate cancer cells

- Preliminary analysis of hormone-resistant human prostate tumors that express high levels of Protocadherin-PC (cytoplasmic form) demonstrate coordinate dysregulation of β -catenin protein (found predominantly in the cytoplasm and nucleus instead of on the membrane)
- The complete cDNA encoding Protocadherin-PC has been cloned and inserted into eukaryotic expression vectors in anticipation of producing LNCaP transformants that express the Protocadherin-PC protein

Reportable Outcomes

Manuscripts – Chen, M.-W., de la Taille, A., Vacherot, F., Gil-Diez-de-Medina, S., Shen, R., Friedman, R.A., Kitajewski, J., Burchardt, M., Chopin, D.K. and Butyan, R. (2000) The emergence of protocadherin-PC expression during the acquisition of apoptosis-resistance by prostate cancer cells. *Nature Medicine* (Submitted).

Patents, Licenses – None.

Degrees – None.

Cell Lines, Tissues or Serum Repositories – Development of two apoptosis-resistant prostate cancer cell derivatives of the LNCaP lineage, LNCaP-TR and LNCaP-SSR. Eukaryotic expression vectors with cDNA encoding human protocadherin-PC

Informatics – Sequence of Protocadherin-PC cDNA (Submitted to Genbank in conjunction with manuscript to *Nature Medicine*)

Funding Applied For – A Phase II application for completion of this project/funding granted (PC001565 – *Protocadherin-PC and Therapeutic Resistance of Prostate Cancer*, R. Butyan, P.I., submitted May 17, 2000)

Employment/Research Opportunities Applied For – None

Conclusions

This phase I project has allowed the description of a unique and novel mechanism whereby prostate cancer cells can develop therapeutic resistance to hormonal-ablation as well as other therapeutic agents. Our experimentation has demonstrated that acquisition of therapeutic resistance by prostate cancer cells is accompanied by increased expression of a very unusual cadherin-family protein now named Protocadherin-PC. The product of the Protocadherin-PC gene, located on the human Y chromosome, lacks a signal sequence, and, as a result, its translation product is cytoplasmic instead of membrane bound as with other members of the cadherin gene family. Our preliminary experimentation suggests that Protocadherin-PC might stimulate apoptosis resistance of prostate cancer cells by activating signaling through the β -catenin (wnt-signaling) pathway, a signaling pathway that is also known to be involved in the progression of human colon cancer and melanoma. Moreover, our survey of actual human prostate tumors demonstrates that expression of this cytoplasmic form of Protocadherin-PC is significantly correlated with the hormone-resistant phenotype and disturbances of the β -catenin signaling

pathway. These results suggest that Protocadherin-PC and the β -catenin signaling pathway might be important targets for the development of therapeutics against hormone-resistant prostate cancer.

References

1. Suzuki, S.T. Structural and functional diversity of cadherin superfamily: are new members of cadherin superfamily involved in signal transduction? *J. Cell Biochem.* **61**, 531-542 (1996).
2. Stappert J. & Kemler, R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* **2**, 319-327 (1994).
3. Aoki, M., Hecht, A., Kruse, U., Kemler, R. & Vogt, P.K. Nuclear endpoint of Wnt signaling: neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. *Proc. Natl. Acad. Sci., U.S.A.* **96**, 139-144 (1999).
4. Moon, R.T. & Miller, J.R. The APC tumor suppressor protein in development and cancer. *Trends Genet.* **13**, 256-258 (1997).
5. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K.W. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790 (1997).
6. Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. & Clevers, H. Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* **18**, 1248-1256 (1998).
7. Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H. & Matsumoto, K. The TAK1-NLK-MAPK-related pathway antagonizes signaling between beta-catenin and transcription factor TCF. *Nature* **399**, 798-802 (1999).
8. Diez de Medina, S.G., Chopin, D., El Marjou, A., Delouvee, A., LaRochelle, W.J., Hozneck, A., Abbou, C., Aaronson, S.A., Thiery, J.P. & Radvanyi, F. Decreased expression of keratinocyte growth factor receptor in a subset of human transitional cell bladder carcinomas. *Oncogene* **14**, 323-330 (1997).